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# Delayed Insemination Results in Embryo Mortality in a Brooding Ascidian

J. STEWART-SAVAGE[1,*], AIMEE PHILLIPPI[2], AND PHILIP O. YUND[2]

[1]*Department of Biological Sciences, University of New Orleans, New Orleans, Louisiana 70148; and*
[2]*Darling Marine Center, School of Marine Sciences, University of Maine, Walpole, Maine 04573*

**Abstract.** We explored the effects of temporal variation in sperm availability on fertilization and subsequent larval development in the colonial ascidian *Botryllus schlosseri*, a brooding hermaphrodite that has a sexual cycle linked to an asexual zooid replacement cycle. We developed a method to quantify the timing of events early in this cycle, and then isolated colonies before the start of the cycle and inseminated them at various times. Colony-wide fertilization levels (assayed by early cleavage) increased from zero to 100% during the period when the siphons of a new generation of zooids were first opening, and remained high for 24 h before slowly declining over the next 48 h. Because embryos are brooded until just before the zooids degenerate at the end of a cycle, delayed fertilization might also affect whether embryos can complete development within the cycle. Consequently, we also determined the effect of delayed insemination on successful embryo development through larval release and metamorphosis. When fertilization was delayed beyond the completion of siphon opening, there was an exponential decline in the percentage of eggs that ultimately produced a metamorphosed larva at the end of the cycle. Thus, even though the majority of oocytes can be fertilized when insemination is delayed for up to 48 h, the resulting embryos cannot complete development before the brooding zooids degenerate.

## Introduction

Field experiments have contributed greatly to current understanding of fertilization processes in free-spawning marine invertebrates (reviewed by Levitan and Petersen, 1995; Yund, 2000). In response to the evidence of potential sperm limitation reported in some field studies, many laboratory studies have started to explore diverse related aspects of invertebrate reproductive biology such as gamete viscosity (Thomas, 1994a,b), egg size and sperm swimming speed (Levitan, 1998), egg longevity (Meidel and Yund, 2001), sperm morphology (Eckelbarger *et al.*, 1989a,b), and the kinetics of fertilization (Young, 1994; Levitan, 1998; Powell *et al.*, 2001). However, results from laboratory studies have in turn led some authors to question the extent to which simple field fertilization experiments adequately mimic the details of fertilization processes in nature (*e.g.*, Thomas, 1994a,b; Meidel and Yund, 2001). Field experiments may often circumvent aspects of reproductive strategies that have evolved to mitigate sperm limitation (Yund, 2000). Hence laboratory experiments still play a vital role in understanding reproductive strategies, and field fertilization studies should endeavor to incorporate the details of the fertilization process gleaned from laboratory work.

Performing realistic field experiments with marine invertebrates that brood embryos presents challenges that are very different from those faced when dealing with broadcast spawners. The biggest challenge with field fertilization studies of broadcasters is interpreting results obtained by artificially holding eggs in a concentrated group (*e.g.*, Levitan and Young, 1995; Wahle and Peckham, 1999) or by removing them from the water column after only a brief interval (Levitan, 1991; Coma and Lasker, 1997). This issue is moot with brooders, who by definition retain eggs and have internal fertilization. However, a different set of problems merits further consideration. The precise timing of egg viability, sperm release, and fertilization itself is often less well understood than in broadcasters. Sperm function may be regulated by the female through sperm chemotaxis (Miller, 1985), activation (Bolton and Havenhand, 1996), or storage (Bishop and Ryland, 1991). In the latter case, the

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* To whom correspondence should be addressed. E-mail: jssavage@uno.edu

temporal pattern of fertilization within a female may be uncoupled from the pattern of sperm release by males. In hermaphrodites, the potential for self-fertilization is a concern, and genetic analyses of paternity may be required to conclusively exclude selfing in some taxa (Yund and McCartney, 1994). For some brooders, the actual path of sperm access to eggs is poorly understood. Information on all of these topics is critical both to the design of more realistic field fertilization studies and to the interpretation of existing studies.

The colonial ascidian *Botryllus schlosseri* is a useful model for field fertilization studies (Grosberg, 1991; Yund and McCartney, 1994; Yund, 1995, 1998). Fertilization is internal, and embryos are brooded until released as tadpole larvae (Milkman, 1967). When colonies are grown on glass surfaces, egg production can be quantified non-destructively (Yund *et al.*, 1997), thus permitting estimation of fertilization levels by comparing egg and embryo counts (Yund, 1995, 1998). Although the general time of fertilization within the life cycle (*i.e.*, temporal resolution on the order of a day) has long been known (Milkman, 1967), the finer-scale timing (temporal resolution on the order of hours) has not been explored. Many authors have assumed that the apparent temporal separation of fertilization and sperm release prevents self-fertilization (*e.g.*, Milkman, 1967; Grosberg, 1987; Yund and McCartney, 1994), but we have recently shown (Stewart-Savage and Yund, 1997) that sperm release commences several days earlier than previously thought. Although sperm storage has been demonstrated in another colonial ascidian (Bishop and Ryland, 1991; Bishop and Sommerfeldt, 1996), past workers have implicitly assumed that storage is unlikely in *B. schlosseri* (Milkman, 1967; Grosberg, 1991; Yund, 1995, 1998). To the best of our knowledge, this assumption has never been explicitly tested. To address this interrelated set of issues, this paper explores the effect of variation in the timing of fertilization on fertilization levels and subsequent larval development in *B. schlosseri*, and compares those results with published information on the timing of sperm release.

## Materials and Methods

### *Study organism*

Colonies of *Botryllus schlosseri* are composed of asexually produced zooids arranged in clusters, or systems, with all zooids in a system sharing a common exhalant siphon. Throughout the life of a colony, all zooids periodically undergo a synchronous asexual zooid replacement cycle in which a new generation of zooids, termed buds, forms between the existing zooids (Berrill, 1941; Izzard, 1973). At the end of the life span of adult zooids (about 8 days at 16 °C; cycle length is temperature dependent), the buds expand, take over the function of the previous generation of zooids (which are quickly resorbed), and then commence their sexual reproductive cycle. The sexual cycle includes the internal fertilization of the mature eggs soon after the inhalant siphons open (Milkman, 1967); the continuous release of sperm starting 16 h later (Stewart-Savage and Yund, 1997); and the brooding of developing embryos, which are released just before the zooids degenerate at the end of the cycle (Milkman, 1967).

### *Standard methods*

The colonies of *B. schlosseri* that were employed in this study were collected from the Damariscotta River, Maine. Animals were grown on glass microscope slides in the flowing seawater system at the University of Maine's Darling Marine Center. Field-collected colonies that had been established in laboratory culture were divided to provide clonal replicates (ramets) of genotypes. Colonies employed in all experiments were monitored for the approach of takeover (the transition between zooid generations). When colonies were about to commence takeover (late stage 5 through early stage 6 by the criteria of Milkman, 1967), they were isolated in 50 ml of sperm-free (aged >24 h) seawater. Isolated colonies were housed in an incubator at 16 °C (range: 14–18 °C) and fed phytoplankton (*Duniella* sp.) at densities of approximately $10^5$ cells/ml. Water and food were changed twice daily. Colonies were monitored for siphon opening and then isolated in individual 250-ml containers with algae (water and food were changed daily) until exposed to sperm. Sperm exposure was accomplished by placing colonies in a flowing seawater tank in proximity to numerous male-phase colonies (>24 h after siphon opening; Stewart-Savage and Yund, 1997) for 1 h. After insemination, colonies were rinsed with aged seawater and returned to isolation.

### *Experimental protocols*

To standardize insemination times, we first had to accurately quantify the start of the reproductive cycle (*i.e.*, the functional opening of siphons). Inhalant siphons are formed early in the takeover process, but the common exhalant siphon of a system generally does not form until near the end. However, it is difficult to ascertain functional siphon opening on morphological criteria alone. In the course of other work, we observed that the consumption of green algae immediately turned the digestive systems of actively feeding zooids (*i.e.*, those that must have open siphons) green. Consequently, we used algal uptake as an assay for siphon opening. To establish the temporal pattern of siphon opening, we isolated 14 colonies and briefly exposed them to algae three to four times during the process of takeover. At each sample interval we recorded the percentage of siphons that were open (% of zooids with green digestive systems). From these data we calculated an average rate of siphon opening. This approach subsequently allowed us to

make single observations of the percentage of siphons that were open and back-calculate the time of the first siphon opening. Both of our other experiments use this approach to estimate the time of initial siphon opening, and the timing of insemination is expressed relative to this event.

To examine the effect of the timing of fertilization on fertilization levels, we exposed colonies to sperm through a range of different times after siphon opening (0.5 to 96 h; $n = 79$). Colonies with about 20 eggs (mean of 20.0 ± standard error of 11.6) were utilized throughout, and all eggs and embryos in a colony were surgically removed 10–18 h after insemination and scored for successful development. Initial studies indicated that embryos should be in the 8-cell to the 32-cell stages during this time range. Uncleaved eggs were scored as unfertilized, as were embryos with an abnormal cleavage pattern (arrested cleavage, abnormal cell number or shape). A few embryos at advanced developmental stages (*e.g.*, gastrula) were excluded from the data set since fertilization was by either contaminating or self sperm.

To examine the effect of timing of fertilization on subsequent development and metamorphosis, colonies were initially fertilized in sets of multiple ramets per genotype. For each genotype, one ramet was left unfertilized (to assess the level of sperm contamination or self-fertilization), one ramet was fertilized about 22 (±2) h after the beginning of siphon opening (when results from the previous experiment indicated that all siphons should be open), and remaining ramets (2–3) were fertilized at various times up to 85 h after initial siphon opening. Because fertilization was consistently minimal in unfertilized controls and the availability of genotypes with multiple egg-bearing ramets was often limited, later trials were conducted without the control treatment. Before takeover, we counted the number of eggs produced by each colony (minimum egg production was set at 25 eggs). After insemination, colonies were returned to isolation until all ramets of a genotype had been fertilized and at least 24 h had elapsed since the last insemination. Colonies were subsequently housed in a flowing seawater table with an independent seawater supply while embryonic development proceeded; they were re-isolated at stage four (Milkman, 1967). After each isolated colony had started the next reproductive cycle, all metamorphosed juveniles in the isolation container were counted. Data from colonies that died or became visibly unhealthy during the experiment were discarded.

## Results

### *Timing of siphon opening*

Feeding did not begin until after the organization of zooids into new systems and formation of the common exhalant siphon. Although the rate of siphon opening varied among colonies (Fig. 1; range of 3.0%/h–17.8%/h), the average rate of siphon opening of the colonies was 7.8%/h ± 4.5%/h ($\bar{X}$ ± SD). We used the average rate of siphon opening to normalize the time of sperm exposure to the start of siphon opening for colonies in the other two experiments.

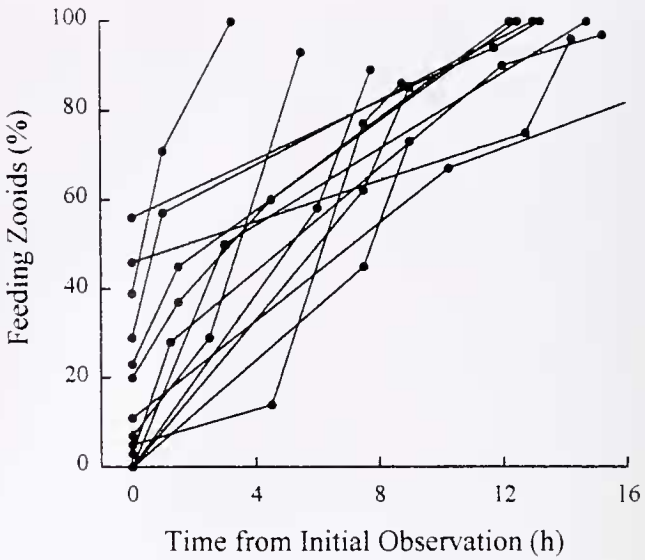


**Figure 1.** Rate of siphon opening in colonies of *Botryllus schlosseri* as assayed by the presence of algae in the digestive system. Colonies were isolated in 50 ml aged seawater with $2 \times 10^5$ algae/ml and monitored at intervals of from 1 to 12 h. Zero time is the first observation of algae in the gut. Temporal patterns for 14 individual colonies are shown. Differences in the *y*-intercept simply reflect how far the takeover process had proceeded when colonies were first observed; slopes indicate the rate of siphon opening.

### *Effect of timing of insemination on fertilization levels*

To determine the time frame during which eggs can be fertilized within the female, we exposed virgin females to a 1-h pulse of sperm at various times after the beginning of siphon opening and assayed successful fertilization by the percentage of normally cleaved embryos present (Fig. 2). When virgin females were exposed to sperm during the period in which their siphons were opening (first 24 h), the level of fertilization increased with time (Fig. 2B). In colonies fertilized during siphon opening, there was no spatial relationship between fertilized and unfertilized eggs either within or among systems; it was common to find both in the same zooid. Because the rate of increasing fertilization (5.4%/h) is similar to the rate of siphon opening (7.8%/h ± 4.5%/h), we conclude that fertilization of the eggs within a zooid occurs shortly after the opening of the siphon.

After the completion of siphon opening, fertilization success remained high (>90%) for 24 h and then declined over the next 48 h with a $T_{50\%}$ of 72 h (Fig. 2B). In a subset of genotypes where multiple ramets were inseminated at different times in the same reproductive cycle, thus controlling for potential genotype and cycle effects, the effect of

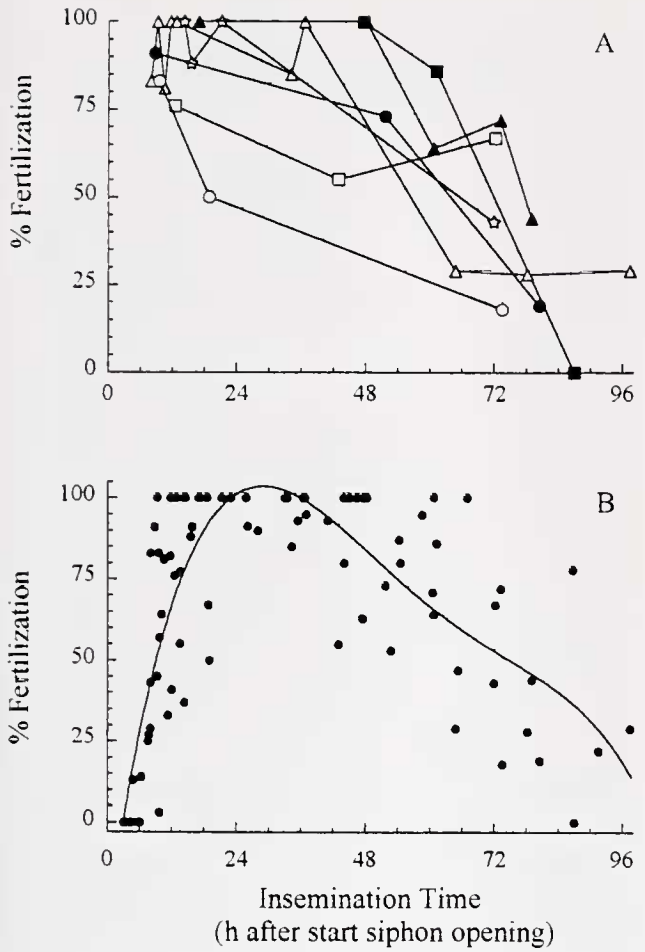


**Figure 2.** Effect of insemination pulse timing on fertilization levels. Colonies were isolated before the start of siphon opening, monitored for the timing of siphon opening, and exposed to sperm for 1 h; the number of cleaving embryos was determined 10–18 h later. (A) Fertilization levels in different ramets of seven genotypes fertilized at different points in the same reproductive cycle. (B) Overall effect of insemination time on fertilization success in ramets from 25 genotypes. The line represents a polynomial regression of the data ($R^2 = 0.580$).

delayed insemination on fertilization varied by genotype (Fig. 2A). Of the seven genotypes in which different ramets were inseminated at different times, five genotypes had a decline in fertilization that mirrored the population data. In the other two genotypes, fertilization levels declined rapidly in one, but remained relatively stable over 60 h in the other. Excluding the genotype that exhibited little decline in fertilization, the average $T_{50\%}$ for the reduction of fertilization was 62 ± 15 h, a value similar to the population-wide regression.

*Effect of timing of insemination on embryo development and metamorphosis*

The maximum duration of gestation is fixed by the length of the asexual zooid replacement cycle. Since eggs could be fertilized well after siphon opening, but the time of embryo release is fixed, we examined the effect of delayed insemination on reproductive success. Successful embryo metamorphosis was selected as an assay of reproductive success because it integrates possible effects on fertilization, development, larval behavior, and settlement. In five trials that included unfertilized (low control), insemination at 22 h (high control), and ramets inseminated at different times after siphon opening, the percentage of eggs that successfully developed through metamorphosis consistently decreased with the time of insemination (Fig. 3A). The unfertilized controls resulted in either zero or very low (<5%) levels of larval metamorphosis (Fig. 3A). However, the percent of eggs developing through metamorphosis varied substantially among 22-h insemination controls (Fig. 3A). Because of the low levels of successful metamorphosis in two genotypes fertilized at 22 h, we calculated the $T_{50\%}$ relative to the maximum value for each genotype. The relative $T_{50\%}$ for the reduction of metamorphosis success was 41 ± 6 h after the start of siphon opening (about 19 h after the completion of siphon opening). When data from all 12 trials were combined (Fig. 3B), larval metamorphosis exhibited an exponential decline with fertilization time beyond 22 h. No larval metamorphosis occurred when colonies were fertilized more than 78 h after the start of siphon opening.

Two outliers (both ramets of the same genotype) had disproportionately high levels of metamorphosis when fertilized about 48 h after siphon opening (Fig. 3B, open squares). Independent evidence (*i.e.*, observations of successful embryo development in isolated colonies) suggested that this genotype may sometimes be able to self-fertilize. Alternatively, the high fertilization levels in these two colonies may be the result of sperm contamination. Because these inconsistent values are limited to one genotype, we have excluded these values from the regression in Figure 3B. Inclusion of the two points in the regression has little effect on the equation parameters, but it substantially reduces the coefficient of determination. Note that many other ramets of this genotype were employed in this experiment (Fig. 3B, open squares) and produced results consistent with those of the other genotypes.

## Discussion

Although more than 50% of *Botryllus schlosseri* eggs can be fertilized 38 to 48 h after the completion of siphon opening (Fig. 2), few viable larvae are produced unless fertilization occurs within the first 19 h (Fig. 3). The decrease in embryo production after delayed fertilization could be caused by either egg aging or limitations on the duration of brooding. As in most invertebrates, the time required to complete development is a function of temperature in *B. schlosseri*. Since the asexual zooid replacement

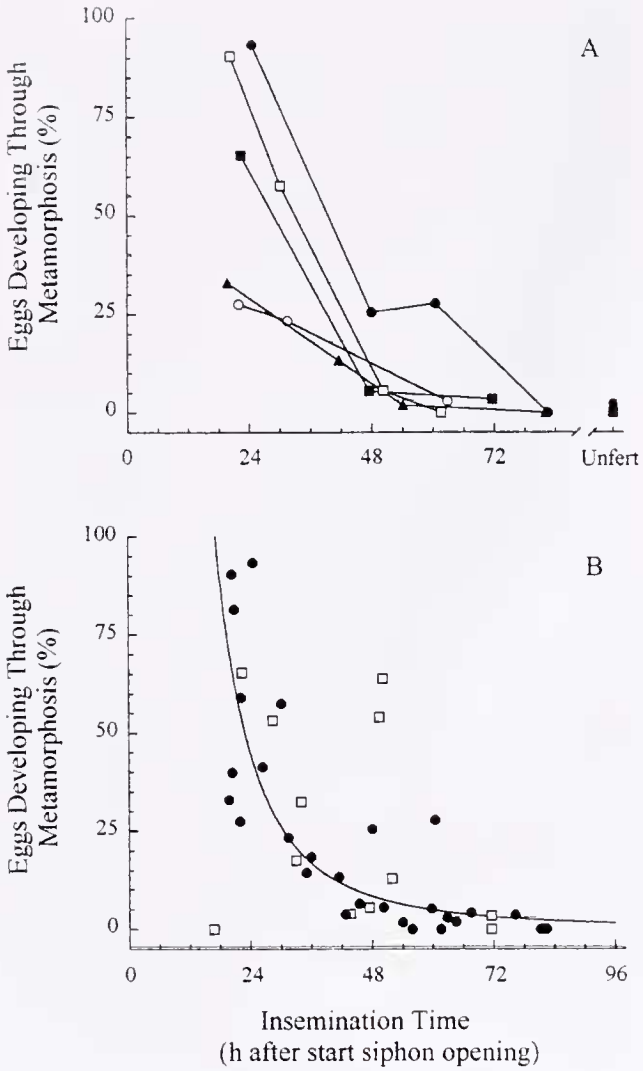


**Figure 3.** Effect of insemination pulse timing on embryo development and larval metamorphosis. Colonies with quantified egg production were isolated before the start of siphon opening, monitored for the timing of siphon opening, and exposed to sperm for 1 h; the number of settled juveniles was determined 5–7 days later. (A) Developmental success of different ramets from five genets. In three of the genets, one ramet was never exposed to sperm (unfertilized, solid symbols). (B) Overall effect of insemination time on successful development. The open squares are the ramets from the putative self-fertilizing genotype; closed symbols represent the other 11 genotypes. The line is an exponential regression of the data except for two outliers at 48 h ($R^2 = 0.713$).

cycle is also a function of temperature (Grosberg, 1982), delayed fertilization could cause the brooding zooids to degenerate before the embryos have become competent to undergo metamorphosis. The deleterious effects of egg aging have been demonstrated in mammals (Juetten and Bavister, 1983; Xu *et al.*, 1997), but such effects are usually manifested early in development. Since early development was normal in all but one colony with delayed fertilization (pers. obs.), the decreased gestational duration caused by delayed fertilization is more likely to be responsible. Nevertheless, additional work on the mechanism by which delayed fertilization decreases larval production could more fully resolve this issue.

In spite of the narrow temporal window in which both fertilization and development are likely to be successful (Figs. 2 and 3), field experiments indicate that colonies of *B. schlosseri* are very adept at acquiring sperm. A single male-phase colony can fertilize most eggs of a nearby female-phase colony with very few sperm (Yund, 1998). If several males are present, they compete to fertilize eggs (Yund, 1995, 1998), and closer males can be successful at the expense of more distant males (Yund and McCartney, 1994). Although sperm transfer usually occurs among nearby colonies (Yund, 1995), sperm can also be obtained from very distant locations when insufficient local sperm are available (Yund, 1998). Even eggs of colonies isolated from the nearest natural populations by tens of meters can be fertilized at appreciable levels (Yund and McCartney, 1994). The apparent ease of fertilization under field conditions, in spite of a very limited temporal window for successful fertilization and development, suggests that the process of sperm capture by colonies must be extremely efficient. Nevertheless, in low-density populations where sperm may be in short supply (Yund, 1998), or in marginal habitats in which sperm production is suppressed (Stewart-Savage *et al.*, 2001), our work suggests that reproductive failure may occur in spite of successful fertilization if fertilization occurs too late in the reproductive cycle. Recent field sampling has demonstrated this phenomenon in natural populations near the end of the annual reproductive season (Yund and Phillippi, unpubl. data).

Unlike the colonial ascidian *Diplosoma listerianum*, in which fertilization can be temporally disassociated from sperm exposure and colonies can store sperm for up to one month (Bishop and Ryland, 1991; Bishop and Sommerfeldt, 1996), *B. schlosseri* colonies apparently cannot store sperm. The evidence for this conclusion is, first, that colonies isolated in sperm-free seawater were not fertilized until we experimentally supplied a sperm pulse, indicating that sperm are not stored and transferred from one asexual generation of zooids to the next. The apparently complete resorption of all zooid tissue at the end of the cycle further suggests that transmission between cycles is unlikely. Second, the tight temporal relationship between siphon opening and fertilization (Fig. 2B) suggests that sperm cannot enter until the new generation of zooids opens its siphons and starts to feed. Third, the narrow window of time in which fertilization is both possible (Fig. 2) and results in viable offspring (Fig. 3) eliminates any apparent fitness advantage to sperm storage within a single asexual generation.

The route of sperm access to eggs in *B. schlosseri* is unknown, but there are at least two possible points of entry (Ryland and Bishop, 1993): sperm enter through the

inhalant siphon and cross the pharyngeal basket to reach the eggs, or sperm enter through the exhalant siphon and then swim to the eggs. During takeover in *B. schlosseri*, the exhalant siphon of each system is formed before the inhalant siphons of all of the component zooids open, and the precise timing of exhalant siphon formation varies among systems (pers. obs.). If sperm enter *via* the exhalant siphon, fertilization levels in the early time intervals of our fertilization timing experiment should have varied among systems, but should not have varied within a system. However, we routinely found mixtures of fertilized and unfertilized eggs within the same system, suggesting that sperm entry to each zooid required an open inhalant as well as exhalant siphon. Although further work is required to determine the route of sperm entry into *Botryllus* colonies, we think it is unlikely that sperm enter *via* the exhalant siphon.

Hermaphroditism creates another challenge for successful reproduction in *B. schlosseri*. Inbreeding depression (Sabbadin, 1971) is likely to exert selective pressure to prevent self-fertilization, even though selfing would be a possible mechanism to assure fertilization in the narrow time window in which fertilization can produce functional embryos. When the data in this paper are combined with previous data on the timing of sperm release (Stewart-Savage and Yund, 1997), it is apparent that the male and female phases of the reproductive cycle overlap in *B. schlosseri* (Fig. 4). Sperm release overlaps for about 48 h with the window for successful fertilization, but there is substantially less overlap with the narrower window in which fertilization results in viable embryos (Fig. 4). Consequently, *B. schlosseri* is not a true sequential hermaphrodite (Milkman, 1967), but the male and female phases are functionally separated in time. This functional segregation of the reproductive phases probably plays some role in ensuring that few metamorphosing embryos result from self-fertilization. However, the very success of our experimental protocols indicates that one or more additional mechanisms to prevent self-fertilization must exist. Eggs of colonies isolated in small volumes of water until points in the reproductive cycle at which substantial self-sperm should have been present (Fig. 4) nevertheless remained unfertilized until we introduced a pulse of sperm (with the possible exception of the two outliers in Fig. 3B). Consequently, some form of self-incompatibility, as described in other colonial and solitary ascidians (Rosati and De Santis, 1978; Bishop, 1996), appears likely in *B. schlosseri* (see also Scofield *et al.*, 1982).

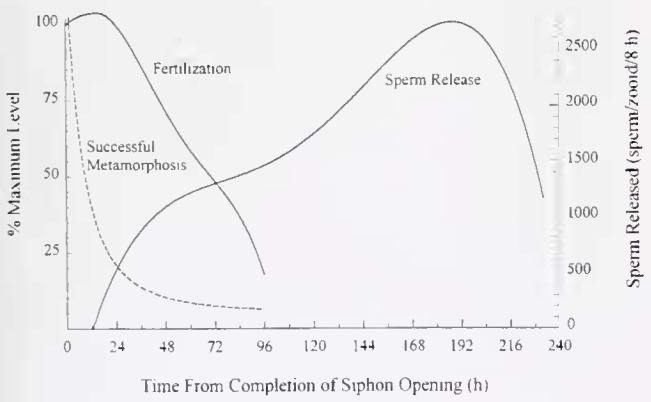


**Figure 4.** Relationship between male and female reproductive phases in *Botryllus schlosseri*. Data collected at different temperatures have been normalized to a 10-day cycle length. The zero time point is the completion, rather than the initiation (as in Figs. 2 and 3), of siphon opening. The sperm release curve is redrawn from Stewart-Savage and Yund (1997) with permission.

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